BioControl (2006) 51:679–701
DOI 10.1007/s10526-005-5271-1

Molecular and morphological examination of *Cyrtobagous* sp. collected from Argentina, Paraguay, Brazil, Australia, and Florida

Paul. T. MADEIRA[1,*], Phil W. TIPPING[1], Daniel E. GANDOLFO[2], Ted D. CENTER[1], Thai K. VAN[1] and Charles W. O'BRIEN[3]
[1]*USDA, ARS, Invasive Plant Research Laboratory, 3205 College Avenue, Fort Lauderdale, FL, 33314, USA;* [2]*USDA, ARS, South American Biological Control Laboratory, Bolivar 1559 (B1686 EFA), Hurlingham, Buenos Aires, Argentina;* [3]*Center for Biological Control, Florida Agricultural and Mechanical University, Tallahassee, FL, 32307-4100, USA*
**Author for correspondence (e-mail: ptmadeira@saa.ars.usda.gov)*

Received 24 August 2004; accepted 16 November 2005

Abstract. Two members of the floating fern genus *Salvinia* (Salviniaceae), *S. minima* Baker and *S. molesta* Mitchell, have established in the United States. *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), long established on Florida *S. minima*, was released in Texas and Louisiana as a biocontrol agent for both species. Subsequently, sequence analysis of the 28S rRNA D2 expansion domain suggested that the Florida and Brazilian populations (used worldwide for biocontrol) of *C. salviniae* might constitute two cryptic species. In response, the Brazilian weevil was imported from Australia and released instead onto *S. molesta*. We sampled *C. singularis* Hustache and *C. salviniae* from their native ranges in Brazil, Argentina, and Paraguay and sequenced them (D2) along with Australian and Florida samples. The genetic distance between *C. singularis* and *C. salviniae* samples is much greater (almost 5×) than the distance between either the Florida and Brazilian samples or the Brazilian and Argentinean/ Paraguayan *C. salviniae* samples. Since *C. singularis* and *C. salviniae* are cryptic species, the Florida and Brazilian populations (or for that matter Brazilian and Argentinean/Paraguayan) could reasonably be described as demes or ecotypes. Occurrence data indicates that, in parts of their ranges, *C. salviniae* and *C. singularis* are not only sympatric but also feed on the same plant species at the same site. While host adaptation (species preferences) likely occurs within local demes, both species seem capable of adapting to the available resource (*Salvinia* species). Finally, a polymerase chain reaction (PCR) primer was developed to distinguish the Florida and Brazilian/ Australian types.

Key words: biological control, cryptic species, *Cyrtobagous*, host adaptation, *Salvinia*, 28S rRNA D2 expansion domain

Introduction

Salvinia molesta Mitchell (Salviniaceae), a floating fern native to South America, has spread widely throughout the tropics and subtropics during the later half of the twentieth century (Abbasi and Nipaney, 1986). Free of natural enemies, its explosive growth forms mats in lakes, ponds and slow moving rivers impeding traffic, degrading recreational use, changing habitats, and interfering with agricultural, water supply, electrical generation, and other uses. Early attempts at manual, chemical and mechanical control proved temporary and expensive, especially for developing countries. Surveys for biological control agents began in the early 1960s (see Table 1 for "A brief timeline of biological control for *Salvinia molesta*") but were confounded by a lack of knowledge of the true identity and native range of *S. molesta*. Eventually, the use of *Cyrtobagous salviniae* Calder and Sands from the native range of *S. molesta* in Brazil proved widely successful.

Today, *S. molesta* is placed within the *Salvinia auriculata* complex which also includes *Salvinia auriculata* Aublet (which it was originally mistaken for), *Salvinia biloba* Raddi, and *Salvinia herzogii* de la Sota. These four species are characterized by divided but apically joined hairs on the abaxial surface of their leaves which form "egg beater" shapes. The members of the complex can be distinguished by sporocarp shape, the arrangement of the sporangia and the pattern of leaf venation (de la Sota, 1962; Mitchell, 1972; Mitchell and Thomas, 1972; Forno, 1983).

Until recently common salvinia, *Salvinia minima* Baker, was the only member of the genus established outside of cultivation in North America. *S. minima* is easily distinguished from members of the *S. auriculata* complex by divided hairs on the abaxial leaf surface that are free and spreading. Small (1931) first reported *S. minima* as naturalized in Florida in 1928 in the St. Johns watershed, although it was mistakenly described as *S. auriculata*. *S. minima*, during the 1930′s, was subsequently found at sites in Miami, Sarasota, and Gainesville, Florida as well as in Savannah, Georgia (Jacono et al., 2001). The earliest collections from the Florida panhandle were made in 1979, followed by Louisiana in 1980, Alabama in 1982, and Texas in 1992 (Landry, 1981). In Louisiana and Texas it aggressively forms thick mats in waterways, marshes, rice fields and crawfish farms. By comparison, *S. minima* in Florida usually appears at lower densities and mixed with other aquaphytes. The weevil, *C. salviniae* was identified

Table 1. A brief timeline of biological control for *Salvinia molesta*

1961–1963	First surveys were conducted in Trinidad, Guyana, and Northeastern Brazil. Three potential agents were identified: 1. *Paulina acuminata* de Geer, a grasshopper 2. *Samea multiplicalis* Guenee, a moth 3. *Cyrtobagous singularis* Hustache, a Curculionid weevil. All were found on *Salvinia auriculata* and collected in Trinidad.
1969 & 1971	The first releases of *Paulina acuminata* were conducted in Zimbabwe. Subsequently released in Kenya and Zambia (1970), Botswana (1971, 1975), Sri Lanka (1973, 1978), India (1994) and Fiji (1975). It failed to establish in Kenya, Botswana and Sri Lanka. Elsewhere it did not provide control.
1970	*Samea multiplicalis* was first released in Zambia, then in Botswana (1972) and Fiji (1976). It established only in Botswana but failed to provide control. Later collected in Brazil and released in Australia (1981) where it established but failed to provide control.
1971 & 1976	The weevil, *Cyrtobagous singularis*, was released twice in Botswana. Also introduced to Zambia (1971) and Fiji (1976) it established in each country but failed to provide control.
1972	*Salvinia molesta*, the plant causing invasive problems worldwide, was described as a unique species (hybrid) distinct from *Salvinia auriculata*. It was thought to be of possible horticultural origin.
1979	The native range of *Salvinia molesta* was discovered in Southeastern Brazil. *Cyrtobagous singularis* feeding on *Salvinia molesta* was collected.
1981	Brazilian *Cyrtobagous singularis* was released in Australia and achieves dramatic control of *Salvinia molesta* on Lake Moondarra. It was subsequently introduced to at least 15 countries achieving almost universal control.
1985	The successful biocontrol agent was described as a separate species, *Cyrtobagous salviniae*.
From:	Julien, M.H., T.D. Center and P.W. Tipping, 2002. 2: Floating fern *Salvinia*). In: R. Van Driesche, S. Lyon, B. Blossey, M. Hoddle, and R. Reardon (eds), *Biological Control of Invasive Plants in the Eastern United States*. USDA Forest Service Publication FHTET-2002-04, pp. 17–32.

on Florida *S. minima* in 1960 on a Miami collection (voucher Florida Agricultural and Mechanical University) and was referenced in the literature by Kissinger (1966) as *Cyrtobagous singularis*. It was later recognized as the recently described sibling species *C. salviniae* (Calder and Sands, 1985). Jacono et al. (2001) believe the reduced aggressiveness of *S. minima* in Florida is due to the presence of this weevil.

S. molesta was first reported in the United States (1995) at a pond in South Carolina where it was subsequently eradicated (Johnson, 1995). It was subsequently reported in Texas in 1997 (Jacono, 1999). It is now reported it in over 36 drainage basins of Texas, Louisiana, Mississippi, Alabama, South and North Carolina, Georgia, Florida, Arizona, California, and Hawaii (see the Non-indigenous Aquatic Species (NAS) database[1] of the United States Geological Survey). Populations of *S. molesta* in Texas, Louisiana, and Alabama generally exhibit the rapid growth and mat formation typical worldwide prior to introduction and control by *C. salviniae* (Julien and Griffiths, 1998).

The process of developing biological control agents (exploration, prioritization, quarantine, host specificity testing, mass rearing, and distribution) can be long and costly. In 1999, cooperators at the USDA ARS Invasive Plant Research Laboratory in Ft. Lauderdale, Florida believed an opportunity to inexpensively control *S. molesta* populations in Texas and Louisiana existed by introducing *C. salviniae* taken from existing populations in Florida. The initial (1999) Florida weevil introductions met with mixed success (Tipping and Center, 2003). Concurrently, Goolsby et al. (2000) presented molecular evidence of sequence divergence between Florida and Australian/Brazilian *C. salvinia* and suggested the differences indicated separate (cryptic) species. They also speculated that the Florida weevil might be specialized on *S. minima* and poorly adapted to *S. molesta*.

Subsequently, further releases of the Florida weevil onto *S. molesta* were discontinued and weevils imported from Australia. After additional host range testing, a general release permit was obtained for part of western Louisiana and eastern Texas. The Australian/Brazilian weevil was first introduced there October, 2001 with the weevils successfully overwintering despite minimum air temperatures at or below 0°C (Tipping and Center, 2003).

Concurrently, a Randomly Amplified DNA (RAPD) study indicated that Louisiana *S. minima* samples were not substantially different from Florida samples (Madeira et al., 2003). A decision was made to re-target the Florida weevil at a troublesome *S. minima* infestation at Jean Lafitte National Historical Park and Preserve in southern Louisiana. Releases were made there in 2002 and 2003. During July 2003, the first evidence of establishment surfaced with the discovery of a lone newly emerged (teneral, brown) weevil. In August, additional freshly emerged adults, significant larval damage, and reduced biomass in the study squares became apparent. However, over-wintering has not yet been conclusively established.

Perhaps in response to Goolsby et al. (2000), anecdotal opinions flourish in the biocontrol and environmental management communities that the Florida weevil is a *S. minima* specialist. However, one of us (Tipping, unpublished data) disputes this assumption. In feeding and life cycle studies of both weevil types on *S. molesta* the Florida type has actually outperformed the Australian weevil.

Therefore, the first and primary purpose of this study was to clarify the taxonomic status of the Florida and Australian (of Brazilian origin) *C. salviniae* types by molecular analysis. While molecular variation can point out clear differences between types it is more difficult to assign exactly how much molecular variation corresponds to what taxonomic difference, especially since the rate of molecular variation can differ between taxonomic groups. Fortunately, it is well established that *C. singularis* and *C. salviniae*, while cryptic, represent separate species due to their differing feeding strategies (Sands and Schotz, 1985), life cycle strategies (Sands et al., 1986) and, especially, failure to hybridize in the laboratory (Calder and Sands, 1985). Therefore, *C. singularis* provides a useful "within group" metric to better assess whether Florida and Australian (Brazilian derived) types also constitute cryptic speciation. The plan was to collect adult *C. singularis* and *C. salviniae* from their native ranges in Brazil, Argentina, and Paraguay and compare them by sequencing with each other and with Australian and Florida samples. The sequencing results would also be compared with the morphological taxonomic identifications generated using the criteria of Calder and Sands (1985).

Two secondary purposes were also considered. One was to examine the site co-occurrence data for the two *Cyrtobagous* species and the *Salvinia* species on which they were found. The other was to examine the sequence data to see if a useful PCR marker could be developed to distinguish the Florida and Brazilian (Australian) weevils. This marker, in turn, could provide a useful tool for determining the origins (Florida and/or Brazilian) of field collections.

Materials and methods

Collection of Salvinia *specimens and* Cyrtobagous *samples*

Adult *Cyrtobagous* samples from Argentina, Brazil and Paraguay were collected during sampling trips in May and September, 2002. The May (M) sampling surveyed *Salvinia* populations along the Atlantic coast from Uruguay north through Curitiba to the Rio de Janeiro region. The September (S) sampling surveyed the Mesopotamia region of Argentina along both the Parana and Uruguay River regions. It also included a survey from Foz do Iguacu, Brazil west to Asuncion, Paraguay and then south to Argentina.

Salvinia samples were examined at each site for sporocarps and, if present, up to 12 samples were chosen and pressed as specimens. The key of Forno (1983) was used for identification. At every consecutive site where *Salvinia* specimens were taken a consecutive number, M1 through M21 or S1 through S28 was assigned, regardless of whether adult weevils were detected. A sampling device was designed to collect *Cyrtobagous* by submersion while on the road. At each site a 15-l sample of *Salvinia* plants was placed in a 55-l plastic storage container. At the end of each day the sampled *Salvinia* inside each container was submersed using a wire mesh. Immediately after submersing the *Salvinia*, small pieces of plants and floating debris were removed and two to three *Salvinia* plants were allowed to float at the surface (above the mesh) so that as *Cyrtobagous* rose to the surface for air they would gather there. After 12–14 h the floating plants were collected and examined under a microscope for *Cyrtobagous*. During eight earlier test trials the floating plants were collected after 12 h and replaced with fresh plants for an additional 12 h. The increased submersion time only increased the recovery of *Cyrtobagous* by between 0 and 3%. Following collection and examination the weevils were placed into 95% ethanol and stored in coolers. As soon as possible the ethanol preserved specimens were stored in a freezer at −80°C for morphological identification and molecular analysis.

A single weevil was selected from each of the South American sample site collections where *Cyrtobagous* was found for sequencing and morphological analysis. This strategy was adopted to maximize potential diversity over the geographic area sampled given limited sequencing resources. "Additional" weevils found at each site were identified only by morphological analysis. The absence of sequence data from sites S10, S11, S19, S20 and S25 reflects missing data due to poor quality sequences but morphologic identifications are still presented.

Five *C. salviniae* samples originating from different sites in Florida and maintained in tank cultures at the USDA ARS Invasive Plant Laboratory were sequenced: F1, F2 and F3 represent weevils raised on *S. minima*; F4 and F5 were maintained on *S. molesta*.

Australian weevils descended from Joinville, Brazil populations were collected at the Wappa Dam, Queensland and shipped by the USDA ARS Australian Biological Control Laboratory to the USDA ARS Invasive Plant Research Laboratory in Florida on July of 2000 for culture and release on *S. molesta*. Five randomly collected individuals from the USDA colony were sequenced (samples A1–A5).

DNA extraction, morphological examination, PCR amplification and sequencing

Three legs were removed from one side of a specimen, ground using a mortar and pestle, and the DNA extracted using the DNeasy Tissue Kit (Qiagen[2]). The remainder of the insect was returned to 95% ethanol in a freezer for morphological taxonomic identification. Morphological examination was conducted by one of us (O'Brien) on both the molecular and "additional" samples using the diagnostic characters described by Calder and Sands (1985).

PCR amplification reactions contained 1× reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO4$, 20 mM Tris–HCl, 2 mM MgSO4,1% Triton X-100), 0.5 mM Betaine, 0.2 mM dNTPs, 0.5 μM each primer and 0.04 units Taq / μl reaction (New England Biolabs[2]). PCR products for sequencing were generated using the "D2F" (CGT GTT GCT TGA TAG TGC AGC) and "D2R" (TTG GTC CGT GTT TCA AGA CGG) universal primers flanking the D2 expansion domain, the least constrained of the 28S rRNA gene expansion segments (Campbell et al., 1993; Goolsby et al., 2000; Gillespie et al., 2005). Reaction tubes were added to a preheated (94 °C) block, incubated at 94 °C for 3 min, then cycled 35 times (denaturation: 94 °C, 1 min; annealing: 55 °C, 1 min; extension: 72 °C, 1.5 min) followed by a final extension (72 °C, 5 min). Amplification products were electrophoresed on 1.4% agarose gels and visualized with ethidium bromide to check for amplification. PCR products were purified using the Qiaprep 96 Turbo Kit (Qiagen[2]) using a Bio-Robot 9600 (Qiagen[2]). Approximately 25 ng of purified PCR products was used as template for sequencing. Sequencing reactions were performed using the Big-Dye Terminator Cycle Sequencing Reaction Kit (Applied

Biosystems[2]) in a 10 μl reaction volume. Both primers "D2F" and "D2R" were used as sequencing primers. Cycle-sequencing products were precipitated using four volumes of 70% isopropanol for 30 min, pelleted, washed with 70% ethanol, re-centrifuged, dried, and the pellet re-suspended in 15 μl of sterile water. The product was then loaded onto a DNA analyzer 3700 (Applied Biosystems[2]).

Alignment and analysis

Sequences were edited using Sequencher 4.1.4 (Gene Codes[2]) and aligned using Clustal X (Thompson et al., 1997) [gap opening = 4, gap extension = 1, DNA transition weight = 0.5]. Parsimony and Maximum Likelihood analysis was conducted using PAUP 4.0b10 (Swofford, 2001). Gaps are treated as "missing". Parsimony analysis was carried out using Branch-and-bound. Multistate taxa were interpreted as uncertainty. Identical taxa (sequences) were identified as a single OTU. Initial upper bounds are computed heuristically. Addition sequence used was simple. Initial 'MaxTrees' setting was auto-increased by 100. Branches were collapsed (creating polytomies) if maximum branch length was zero. The 'MulTrees' option was in effect.

Maximum Likelihood was examined starting with default settings including empirical nucleotide frequencies. Substitution types (1, 2, 6) corresponding to the F81 model, the HKY85 model, and the GTR model were compared. For each model the initial rates were set to 1. The rate matrix generated was then input (lset rmatrix = est;) and likelihood rerun. Finally, the rate variation across sites (Gamma shape) was estimated (lset rates = gamma shape = estimate;) and likelihood rerun. This optimization was repeated one additional time. These settings correspond to the F81 + G, HKY85 + G model and GTR + G models.

Matching previous alignment and developing PCR "Type" assay

In order to compare results with those reported earlier (Goolsby et al., 2000) various gap costs were entered into Clustal X and the results of the alignments compared. The alignment closest to Goolsby et al. (2000) was then examined using PAUP as before to check the redundancy of the taxonomic results when using very different gap costs. Differences in sequences from the Australian (Brazilian) and Florida *C. salviniae* were examined to see if a primer could be designed which would work to amplify one "type" but not the other.

Results

Co-occurrence of Salvinia *and* Cyrtobagous *samples*

Table 2 presents collection site information: sample site numbers, GPS and locality information, the *Salvinia* species found at each site and the number of each *Cyrtobagous* species (identified morphologically) at each site. Figure 1 presents a regional map with collection sites identified by site number. Site number may be used to cross-reference *Cyrtobagous* samples on the map (Figure 1), co-occurrence (Table 2) and molecular data (Figures 2 and 3). Florida and Australian samples are identified in "Materials and methods" and may be cross-referenced to the molecular data (Figures 2 and 3). Note that only the sites where sampling yielded *Cyrtobagous* are displayed (explaining why so many site numbers are absent). During a February 2002 survey and the May (M) 2002 sampling, *Salvinia* was quite common from the Uruguay–Brazil border up to Porto Alegre, and then north of Rio. However, it was difficult to find *Salvinia* in the area of Joinville and Curitiba, where Forno (1983) reported its distribution. No *S. molesta* samples were identified on either of these trips although they have been found there on other trips through the area.

On the May (M) trip *Cyrtobagous* was found only in the northernmost sites, possibly because it was early in the season and colder in the south. Additionally, only *C. salviniae* was identified in the "M" samples around Rio de Janeiro (on *S. biloba*). In contrast, both *Cyrtobagous* species occurred throughout northeastern Argentina and southern Paraguay during the September (S) trip. One of the two sample sites (S16, S20) with only *S. minima* had only *C. salviniae* while the other had both species. The single site containing only *S. herzogii* (S24) also contained only *C. salviniae*. Where *S. herzogii* and *S. minima* co-occurred (S1, S5), only *C. salviniae* was found. There were 10 sites where only *S. auriculata* occurred. At 5 of these sites (S9, S10, S11, S12, S14) only *C. salviniae* occurred, while 4 other sites (S7, S15, S19, S26) had both *C. salviniae* and *C. singularis*, and 1 site (S20) had only *C. singularis*. The distribution of *Cyrtobagous* corresponds well to that reported by Calder and Sands (1985).

Parsimony and maximum likelihood analysis

The sequences generated in this study have been deposited as accession numbers AY819664 to AY819693 in the National Center for Biotechnology Information (NCBI) database.

Table 2. Sample site identification, *Salvinia* and *Cyrtobagous* co-occurance data

Site Number*	GPS latitude	GPS longitude	Country**	State***	Collection Site	*S. minima*	*S. auriculata*cmplx	*S. auriculata*	*S. herzogii*	*S. biloba*	Ided**** # *C. salvinae*	Ided**** # *C.singularis*
M13	S 22°48′	W 42°25′	BR	RJ	RJ124, 8 km W of Araruama		X			X	23	0
M14	S 22°49′	W 42°18′	BR	RJ	14 km S Sao Vicente de Padua		X			X	2	0
M15	S 22°48′	W 22°17′	BR	RJ	11 km S Sao Vicente de Padua		X			X	4	0
M18	S 22°26′	W 41°52′	BR	RJ	RJ106 10 km S Macaé		X			X	5	0
M19	S 21°49′	W 41°30′	BR	RJ	BR101, 22 km S Campos dos Goitacazes		X			X	2	0
M21	S 22°30′	W 42°17′	BR	RJ	BR101, 9 km S Casimiro de Abreu		X			X	1	0
S1	S 33°49′	W 58°52′	AR	ER	Rd 12, 20 km S Villa Paranacito	X	X		X		5	0
S5	S 30°20′	W 59°31′	AR	CR	Rd 12, 1 km N. Entre Ríos border	X	X		X		2	0
S7	S 29°58′	W 59°29′	AR	CR	Rd 12, 12 km N Esquina		X	X			5	2
S8	S 28°45′	W 58°41′	AR	CR	Junction Rd 12 and Rd 118	X	X	X			2	4
S9	S 28°28′	W 58°41′	AR	CR	Tabay		X	X			2	0
S10	S 28°01′	W 57°39′	AR	CR	Rd 118, 48 km SW Loreto		X	X			2	0
S11	S 27°40′	W 57°16′	AR	CR	Loreto		X	X			2	0
S12	S 27°36′	W 57°00′	AR	CR	Rd 12, 26 km W Villa Olivari		X	X			6	0
S14	S 27°25′	W 55°37′	AR	MS	near Santa Ana		X	X			4	0

S15	S 25°08′	W 57°33′	PY	CN	Remanso		X	X		9	7
S16	S 25°01′	W 57°33′	PY	VH	1 km W Villa Hayes	X				2	1
S17	S 25°10′	W 57°35′	PY	CN	near Remanso bridge	X	X	X		2	4
S19	S 25°10′	W 57°44′	PY	VH	12 km W J. Falcon		X	X		1	7
S20	S 25°14′	W 57°40′	PY	VH	5 km E J Falcon		X	X		0	2
S21	S 26°24′	W 57°07′	PY	MS	Villa Florida, Tebicuary River	X	X	X		6	4
S22	S 26°27′	W 57°05′	PY	MS	7 km SE Villa Florida	X				1	0
S24	S 26°33′	W 57°03′	PY	MS	3 km SE San Miguel		X		X	3	0
S25	S 28°46′	W 56°22′	AR	CR	Rd 14, 34 km S. Santo Tome		X		?	0	3
S26	S 29°01′	W 56°29′	AR	CR	Rd 14, 11 km N Alvear		X	X		2	5
S27	S 29°43′	W 57°05′	AR	CR	Paso de los Libres	X	X	X	X	0	2

Site Number*: M samples collected May 2002 by D. Gandolfo & J. Jara S samples collected September 2002 by D. Gandolfo, F. McKay, & P. Madeira
Country**: AR = Argentina, BR = Brazil, PY = Paraguay
State***: CN = Central, CR = Corrientes, ER = Entre Rios, MS = Misiones, RJ = Rio do Janeiro, VH = Villa Hayes
Ided**** # Identification by morphological features of adult follow Calder and Sands (1985)
S. auriculata complex alone checked where sporocarps not present. "?" indicates uncertainty in ID.

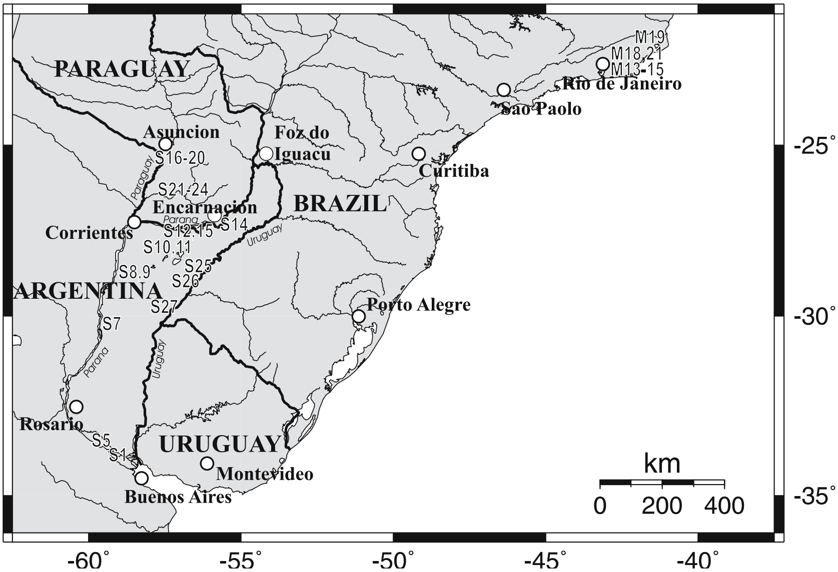

Figure 1. Map of sample sites where *Cyrtobagous* was sampled showing countries, major cities, and major rivers. Sample (site) numbers [M = Brazil (May sampling), S = Argentina /Paraguay (September sampling)] may be cross-referenced with the other figures and table 2.

Of 548 total characters for the aligned sequences, 516 are constant, 27 are parsimony-informative (PI), and 5 are variable but parsimony-uninformative (V/PU). The empirical nucleotide frequencies are A = 0.21978 C = 0.23870 G = 0.29946 T = 0.24206. When *C. singularis* is considered alone, only 1 character is PI while 2 are V/PU. When only *C. salviniae* are considered, 10 characters are PI while five are V/PU. If Florida samples are removed, the remaining *C. salviniae* sequences include 6 PI and 4 V/PU characters. By the same token if only the Brazilian, Australian, and Florida are compared there are also 6 PI and 4 V/PU. When the "M" or "S" *C. salviniae* samples are looked at alone, for each case, only 1 character is PI while 2 are V/PU.

The Branch-and-bound parsimony analysis retained 1188 trees with a "best" tree score of 36 (Sum of min. possible lengths = 34, Sum of max. possible lengths = 152), a consistency index (CI) = 0.944, a retention index (RI) = 0.983, and a rescaled consistency index (RC) = 0.928. The Semi-Strict Consensus Tree generated from the 1188 "best" trees is displayed in Figure 2a. The parsimony consensus numbers are not bounded by parentheses. In 100% of the trees the Florida samples are distinct from the South American *C. salviniae* and *C. singularis* samples. Also, in 100% of the trees, the *C. salviniae* and *C. singularis*

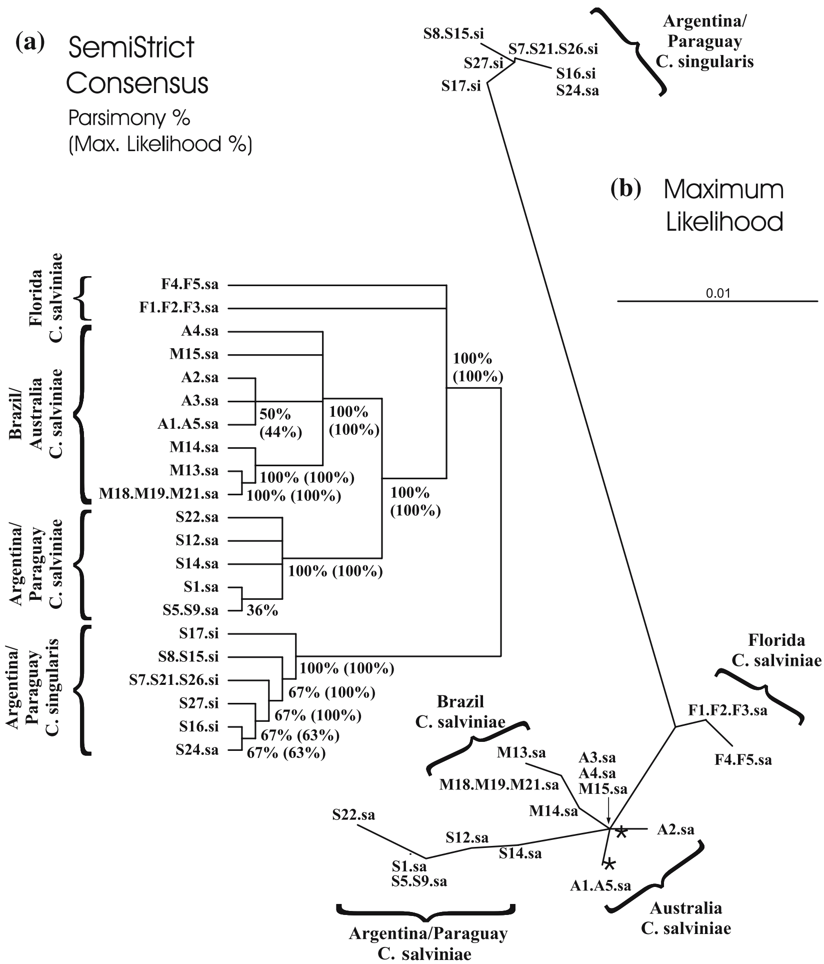

Figure 2. Parsimony and maximum likelihood analysis of D2 expansion domain – 28S rRNA sequences from *Cyrtobagous* **samples**. Sample (site) numbers may be cross-referenced with the other figures and table 2. Sample numbers on the same line separated by "." represent identical sequences. [M = Brazil (May sampling), S = Argentina /Paraguay (September sampling); F = Florida samples, taken from USDA colonies but originally from different Florida sites; A = Australia samples, randomly collected from USDA colony] Morphological identifications follow sample numbers [sa = *C. salviniae*; si = *C. singularis*). Cluster identities indicated by heavy brace "{". (a) SemiStrict Consensus percentages representing the probability that the cluster to the left of the node will occur exactly as shown. % without parentheses: The branch-and-bound parsimony, consensus of 1188 "best" trees. % with parentheses: HKY maximum likelihood, consensus of 16. (a) Maximum Likelihood – one of the two "best" trees generated by the HKY + G analysis is presented. The second "best" tree differs only in that the branch with sample A2.sa shifts to the alternate "*" position next to sample A1.A5.sa. Samples are placed at nodes. Distance between nodes represents sequence divergence.

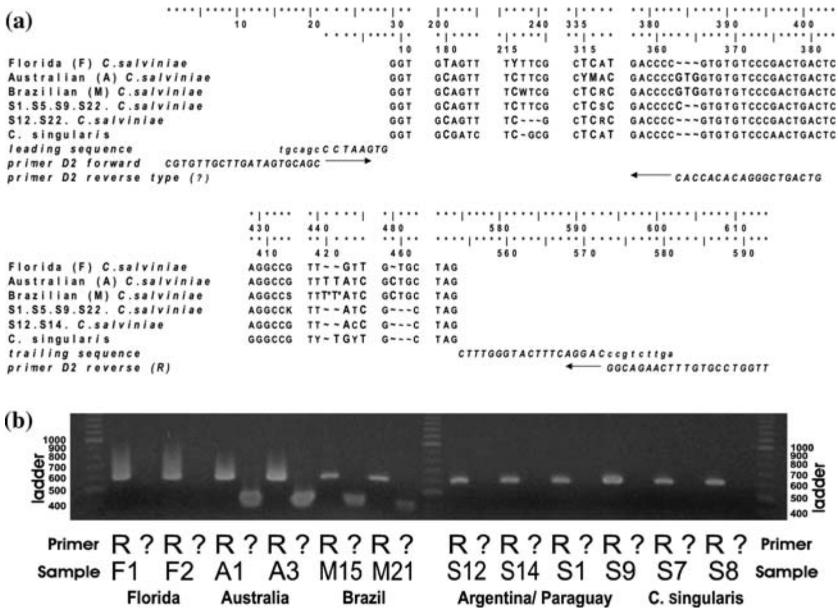

Figure 3. Comparison of Brazilian (Australian) and Florida *Cyrtobagous salviniae* D2 expansion domain – 28S rRNA Sequences with additional reference to other South American *C. salviniae* and *C. singularis*. Sample numbers [F = Florida, A = Australia, M = Brazil (May sampling), S = Argentina/ Paraguay (September sampling)] may be cross-referenced with the other figures and table. (a) Sequence beginning, end, and variable regions for the Australian and Florida types. Positions where Florida and Australian *C. salviniae* samples differ are highlighted by larger a font. A default alignment of Clustal X (gap opening = 10; gap extension = 0.2) with minor manual changes is presented for comparison with Goolsby et al. (2000). Two positional numberings are presented. The first starts with the forward primer. The second begins at position 21 of the first where Goolsby et al. (2000) sequences began. Leading and trailing sequences overlap with the primers and appeared in only some of our samples due to different read starts (indicated by lower case). An indel at 363-5 (lower positional numbering) allowed for design of primer D2 reverse type (?). The "*" at lower position 420-1 indicates one Brazilian sample (M15) had an indel there. Morphological identifications follow sample numbers. (b) Gel of PCRs utilizing the D2 reverse primer (?) diagnostic for Brazilian/Australian *C. salviniae*. D2 standard reverse primer (R) PCRs generated as a positive control. All samples not indicated as *C. singularis* are *C. salviniae*.

samples occur on separate branches indicating that molecular and morphological identities agree (one exception S24.sa appears to be a morphological misidentification).

The F81 maximum likelihood search (substitution type, ST = 1) retained 48 trees with a "best" tree score (−ln L) = 1004.8 . The HKY85 maximum likelihood search (ST = 2) retained 16 trees with

$-ln\ L = 990.7$. The GTR maximum likelihood search (ST = 6) retained 8 trees with -ln L = 988.0 . The F81 + G model (estimated rate matrix and gamma shaped estimate across sites) retained 19 trees with a "best" tree score (−ln L) = 1001.3 . The HKY85 + G model retained 2 trees with −ln L = 987.2 . The GTR + G model retained 2 trees with −ln L = 984.5 . Because GTR + G failed to substantially improve the score over the simpler HKY + G model, or for that matter the HKY model, results from the latter two are presented.

The semistrict consensus tree percentages from the 16 trees generated using the HKY model are presented in parentheses next to the parsimony percentages (Figure 2a). Clearly, as with the heuristic analysis, the Florida population is distinct from both the South American *S. salviniae* and *S. singularis* according to the consensus analysis. However, in both parsimony and maximum likelihood consensus trees the Brazilian (M) *C. salviniae* samples are also distinct from the Argentinean/ Paraguayan (S) samples.

One of the two "best" trees generated by the HKY + G maximum likelihood analysis is presented in Figure 2b. The second "best" tree differs only in that the branch with sample A2.sa shifts to the alternate "*" position next to sample A1.A5.sa. Although the (−ln L) score of the HKY + G trees are not significantly better than the 16 HKY trees, a HKY + G tree was chosen for display under maximum likelihood in order to present graphically the evolutionary distances. It is clear that the distance between the closest *C. singularis* and *C. salviniae* samples is much greater than the distance between the closest Florida and Brazilian *C. salviniae* samples. Further, there is clearly similar distance between the Florida "F" samples and Brazilian "M" samples as between the Brazilian "M" samples and Argentinean/Paraguayan "S" samples.

Comparison of Brazilian (Australian) and Florida C. salviniae *sequences*

In order to contrast the results of this study with that of Goolsby et al. (2000), various alignments were examined and compared to the previous description (since their alignment parameters were not stated). These alignments included *C. singularis* and *C. salviniae* from South America in addition to the Australian and Florida samples so, not surprisingly, the alignments did not perfectly match that given previously. Among the alignments examined the default alignment of Clustal X (gap opening = 10; gap extension = 0.2) with minor manual changes produced the closest match to Goolsby et al. (2000). The heuristic semistrict consensus tree generated by PAUP for this alignment produced identical results to those in Figure 2a. Maximum Likelihood showed minor shifts in the trees with no effect on the conclusions. This "comparison" alignment is presented in Figure 3a. Note that two positional numberings are presented. The first corresponds to base 1 beginning at the start of the forward primer. The second, provided for reference, begins at position 21 of the first, and corresponds to the beginning of the sequences of Goolsby et al. (2000). The leading and trailing sequences presented overlap with the primers and represent sequence which appeared in some but not all of our samples due to different read starts. Only the beginning, ending, and regions where the Australian and Florida sequences differ are presented.

Goolsby et al. (2000) reported one intra-specific polymorphic site in each population, at position 316 for the Australian and at position 216 for Florida. This study found an additional intra-specific polymorphic site at position 315 for the Australian. Goolsby et al. (2000) reported four transitions which distinguish the types, at 180, 318, 412 and 423. This alignment also shows four transitions, however they appear at 180, 318, 422 and 424. Goolsby et al. (2000) report indels at 365-7, 420-1 and 459. In this alignment the 365-7 gap appears at 363-5 but could as easily be placed as before.

Examination of the sequence differences suggested that the indel at 363-5 might allow for a reverse primer capable of distinguishing the types [called primer D2 reverse type (?) in Figure 3a] when combined with the D2 forward primer. This primer should produce a product in the Brazilian (M) and Australian (A) *C. salviniae* samples but not in the Florida (F) and Argentinean/Paraguayan (S) *C. salviniae* or *C. singularis*.

PCR amplification was conducted with the same reaction components and cycle conditions used in the sequencing PCRs except no Betaine was used and the annealing temperature was raised to 56 °C to increase stringency. The master mix contained the D2 forward primer while the reverse primer was added separately to each tube. The D2 reverse primer was used as a positive control in one tube while the D2 reverse type (?) primer was used as the diagnostic in an adjacent tube. Figure 3b presents the PCR results on an agarose gel for representative members of the Florida (F), Australian (A), Brazilian (M), and 2 gap types of (S) *C. salviniae* as well as for *C. singularis* (S7, S8). Amplification using the D2 reverse type (?) primer occurred only in the Australian (A) and Brazilian (M) *C. salviniae* samples as anticipated.

Discussion

Relationship of Salvinia *and* Cyrtobagous *species*

Although sample sizes are too small to draw sweeping conclusions, it appears that *C. salviniae* can utilize *S. biloba*, *S. auriculata* and *S. minima* since it was found on populations of each (Table 2). It also appeared with *S. herzogii*, and was identified on *S. herzogii* plants, although in each case at least one other *Salvinia* was also present. Clearly it also does well on *S. molesta*, as per its success as a biocontrol agent.

C. singularis appeared mostly on *S. auriculata* but did appear on *S. minima* and possibly on *S. herzogii*. It is also well known that while *C. singularis* is not an effective biocontrol agent for *S. molesta*, it has survived and persisted on it (Julien and Griffiths, 1998) in Botswana, Zambia and Fiji. *C. singularis* has also been reported on *S. oblongifolia* in Bahia, eastern Brazil (Calder and Sands, 1985).

This study also found that the range of *C. salviniae* and *C. singularis* is sympatric, both species can occur at the same site and persist on the same *Salvinia* species. While host species preferences may and probably do occur within local demes of each *Cyrtobagous* species, both seem capable of adapting to whichever *Salvinia* species is available. The Florida weevil exemplifies this flexibility. It is smaller than *C. salviniae* from Brazil (Calder and Sands, 1985), probably because its larvae feed primarily by tunneling into the rhizome and *S. minima*, the primary host available in Florida, is smaller than the members of the *S. auriculata* complex.

Sands et al. (1986) showed that, at least on *S. molesta*, the intrinsic rate of increase of *C. salviniae* was much greater than *C. singularis*. Further, the performance of *C. salviniae* was as good or better for all life stages at all temperatures tested. This raises the question of why both species of *Cyrtobagous* persist over their sympatric range. Host adaptation of *C. salviniae* to *S. molesta* may provide a partial explanation of the performance differences. The Australian *C. salviniae* used for the study were collected on *S. molesta* and may be better adapted to that plant than the *C. singularis* collected on *S. auriculata* in Trinidad. In turn, *C. singularis* might show advantages if it was tested on *S. auriculata*. However, the most likely explanation for their co-existence within a sympatric range is the niche differentiation presented by Sands and Schotz (1985). Adults of *C. salviniae* feed mainly on buds while *C. singularis* feed extensively on other plant parts.

C. salviniae larvae tunnel into the rhizomes destroying the vascular bundles while *C. singularis* larvae mainly feed externally. Another fascinating but speculative question is whether this niche differentiation is evidence of sympatric speciation or a coincidence of different allopatric evolutionary paths.

Note that *C. singularis* is the predominant species in the more equatorial region of Calder and Sands' (1985) map. However, because *C. salviniae* appears to do quite well on *S. minima*, as evidenced by Florida and samples S16 and S20, and since *S. minima* has a broad geographic range in South and Central America, it is probable that the range of *C. salviniae* is more extensive than currently identified.

One of the secondary purposes of this study was to see whether a host preference could be detected for the two *Cyrtobagous* species sampled on native *Salvinia* species during collection by examining the site co-occurrence data. This study indicated that *C. salviniae* is established on all the members of the *S. auriculata* complex as well as *S. minima*. This co-occurrence study does not delineate preferences, however, it appears that while *C. singularis* may persist on other *Salvinias*, it is primarily associated with *Salvinia auriculata*.

Molecular comparison of C. singularis *and* C. salviniae

The primary purpose of this study was to clarify the taxonomic status of the Florida and Australian (of Brazilian origin) *C. salviniae* populations. As noted earlier, there is similar genetic distance between the Brazilian "M" and Argentinean/Paraguayan "S" samples as between the Florida and Australian/ Brazilian types. There seems no molecular phylogenetic reason to consider the Florida and Australian/ Brazilian types as separate species. Having said that, the Florida type does group distinctly suggesting its origin is not from the South American areas sampled. Given the probable co-introduction of *C. salviniae* to Florida in one of the *S. minima* introductions and the broad geographic region where *S. minima* occurs, the sequence divergence of the Florida population of *C. salviniae* likely occurs because it originated from a different region of *S. minima's* host range. The variation between the Brazilian "M" and Argentinean/Paraguayan "S" samples, on the other hand, may reflect host differences given their relative geographical proximity. The Brazilian weevils all were found on *S. biloba* while none of the Argentinean/ Paraguayan "S" samples were.

Recall that *C. singularis* was chosen as a molecular yardstick to inform the speciation issue. As indicated previously, the molecular distance between the closest *C. singularis* and *C. salviniae* samples is much greater (almost 5×) than the distance between the closest Florida and Brazilian *C. salviniae* samples. Since *C. singularis* and *C. salviniae* are best described as cryptic species, the Florida and Brazilian (for that matter, between the Brazilian and Argentinean/ Paraguayan) *C. salviniae* might reasonably be described as demes or ecotypes.

Crossing experiments provide perhaps the best, and certainly the most classical test of speciation. Recently, one of us (Tipping, unpublished data) successfully conducted reciprocal crosses of the Florida and Australian/Brazilian types of *C. salviniae*. Further, F1 crosses produced a F2 generation. There now seems little doubt that these types should be considered the same species.

Comparison of Brazilian (Australian) and Floridian C. salviniae *sequences*

A secondary purpose of this study was to examine the sequence data and see if a useful PCR marker could be developed to distinguish Florida and Brazilian (Australian) weevils in field collections. The design of an indel specific reverse primer [called primer D2 reverse type (?) in Figure 3a] capable of distinguishing the types when combined with the D2 forward primer has been presented. A product is produced for the Brazilian (M) and Australian (A) *C. salviniae* samples but not for the Florida (F) and Argentinian/Paraguayan *C. salviniae*. The standard D2 primers may be used as a positive control to assure that the absence of an amplicon is not due to DNA quality issues. These results have been replicated three times with the subset of the samples shown in Figure 3, twice with additional samples not shown, and with a different thermal cycler a year later.

Therefore, in areas where both the Florida and Brazilian types have been released (i.e. Louisiana) this PCR reaction provides a diagnostic tool to determine the presence/ absence of each. However, several qualifications must be made about the use of this tool. First, it is strongly suggested that known control weevils be used to check and optimize the PCR reactions. If false positives appear with Florida weevils try increasing annealing temperature. Second, a sub-sample of the insects double checked by sequencing would further insure reliability. Finally, since it is probable the types will cross propagate where their ranges overlap, heterozygotes would be expected to produce the diagnostic band. Therefore, a fairly large sample must be employed to assure detection of the Florida homozygote. If the sole goal is to detect whether the Florida type contributed to the population this analysis will suffice. If the researcher wishes to estimate the relative contributions of the two types it is necessary to develop a maternally inherited, haploid (mitochondrial) marker or to conduct a more extensive, population genetic study.

Host adaptation, host race formation and speciation

Walsh (1864) first discussed the effects of host plant choice on the formation of different phytophagous insect races. He noted that individual insects often display a great deal of plasticity in host choice behavior and suggested that shifting host preferences might eventually result in new host races. Bush (1969) presented a definition of host race as "a population of a species living on and showing a preference for a host which is different from the host or hosts of other populations of the same species. Host races represent a continuum between forms that freely interbreed to those that rarely exchange genes." At the next level Diehl and Bush (1984) defined host-associated sibling species by the requirement that the biotypes be effectively reproductively isolated. The differences between *C. singularis* and *C. salviniae* are easily placed at this level (sibling species) by their failure to cross and the molecular evidence. How completely the differences are "host associated" is debatable given the range of host/ weevil co-occurrences (within New World *Salvinias*) documented in this survey. Sands et al. (1986) suggest that host adaptation of *C. singularis* to *S. auriculata's* higher nitrogen tissues are partly responsible for its lower performance on *S. molesta*.

Recently, discussions of host race formation have proliferated along side of the increasing data and theoretical mechanisms informing the likelihood of sympatric speciation. Various authors have begun to propose criteria which can be experimentally verified (Jaenike, 1981; Bush, 1992; Berlocher and Feder, 2002; Drés and Mallet, 2002). Few, however, can be verified by this study since it lacks information on criteria such as host performance, host fidelity for specific populations, measures of differentiation at multiple loci, correlation between host choice and mate choice, and higher fitness on natal than alternative hosts. The genetic differentiation which appears in the Brazilian *C. salviniae*, considering the geographic proximity to its Argentinean/ Paraguayan neighbors, may be the result of host differentiation onto *S. biloba* and *S. molesta*. However, it may also merely be the result of a reduction of migration due to geographic separation. Lacking other members of the *S. auriculata* complex within these Brazilian samples, defining this as host differentiation is speculative.

Via (1990), in his review of host adaptation in herbivorous insects stated, "Simply observing that some populations use only a limited subset of hosts used by the species is consistent with use of a locally abundant resource by generalists; Evidence for specialization can only be claimed if transplants reveal poor performance of genotypes or populations on alternate plants." This study indicates that *Cyrtobagous*, especially *C. salviniae*, can be considered a generalist on "New World" *Salvinia* species. Via's (1990) cautionary comment seems applicable in light of the widespread opinion that Florida *C. salviniae* is a *Salvinia minima* specialist. It's best to do the science (host specificity studies, host performance studies, correlation between the geographic source of the invasive species and the natural enemy) rather than making policy decisions based on mere association.

Acknowledgements

The authors gratefully acknowledge Fernando McKay and Javier Jara of the USDA ARS, South American Biological Control Laboratory whose experienced eyes could pick out a *Salvinia* site at 80 km/hr, and who worked late into the night processing samples. Thanks also to Mike Vacek and Lisa Brutcher, Southern Conservation Corps (SCA) volunteers, whose hard work kept the molecular laboratory running. Finally, the authors are grateful to Phat Dang and Randall Niedz of the USDA Horticultural Research Laboratory in Ft. Pierce, Florida for the assistance of their sequencing laboratory.

Notes

1. The Nonindigenous Aquatic Species (NAS) database web site can be accessed at http://nas.er.usgs.gov
2. Mention of a trademark name, proprietary product, or specific equipment does not constitute a warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that also may be suitable.

References

Abbasi, S.A. and P.C. Nipaney, 1986. Infestation by aquatic weeds of the fern genus *Salvinia*: Its status and control. *Env. Cons.* 13: 235–241.

Berlocher, S.H. and J.L. Feder, 2002. Sympatric speciation in phytophagous insects: moving beyond controversy? *Annu. Rev. Entomol.* 47: 773–815.

Bush, G.L., 1969. Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera: Tephritidae). *Evolution* 23: 237–251.

Bush, G.L., 1992. Host race formation and sympatric speciation in *Rhagoletis* fruit flies (Diptera: Tephritidae). *Psyche* 99: 335–355.

Calder, A.A. and D.P.A. Sands, 1985. A new Brazilian *Cyrtobagous* Hustache (Coleoptera: Curculiondae) introduced into Australia to control *Salvinia*. *J. Aust. Ent. Soc.* 24: 57–64.

Campbell, B.C., J.D. Steffen-Campbell and J.H. Werren, 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera:Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28s rDNA sequences. *Insect Mol. Biol.* 2: 225–237.

de la Sota, E.R., 1962. Contribución al conocimiento de las Salviniaceae neotropicales. I. *Salvinia oblongifolia* Martius, II. *Salvinia auriculata* Aublet, III. *Salvinia herzogii* nov. sp. *Darwiniana* 12: 465–520.

Diehl, S.R. and G.L. Bus, 1984. An evolutionary and applied perspective of insect biotypes. *Annu. Rev. Entomol.* 29: 471–504.

Drès, M. and J. Mallet, 2002. Host races in plant-feeding insects and their importance in sympatric speciation. *Phil. Trans. Roy. Soc. Lond. B* 357: 471–492.

Forno, I.W., 1983. Native distribution of the *Salvinia auriculata* complex and keys to species identification. *Aquat. Bot.* 17: 71–83.

Gillespie, J.J., J.B. Munro, J.M. Deraty, M.J. Yoder, A.K. Owen and A.E. Carmichael, 2005. A secondary structural model of the 28S rRNA expansion segments D2 and D3 for Chalcidoid wasps (Hymenoptera: Chalcidoidea). *Mol. Biol. Evol.* 22: 1593–1608.

Goolsby, J.A., P.W. Tipping, T.D. Center and F. Driver, 2000. Evidence of a new *Cyrtobagous* species (Coleoptera: Curculionidae) on *Salvinia molesta* Baker in Florida. *Southwestern Entomologist* 25: 299–301.

Jacono, C.C., 1999. *Salvinia molesta* (Salviniaceae) new to Texas and Louisiana. *Sida* 18: 927–928.

Jacono, C.C., T.R. Davern and T.D. Center, 2001. The adventive status of *Salvinia minima* and *S. molesta* in the southern U.S. and the related distribution of the weevil *Cyrtobagous salviniae*. *Castanea* 66: 214–226.

Jaenike, J., 1981. Criteria for ascertaining the existence of host races. *Am. Nat.* 117: 830–834.

Johnson, D., 1995. Giant *Salvinia* found in South Carolina. *Aquatics* 17: 22.

Julien M.H. and M.W. Griffiths, 1998. *Biological control of weeds. A World Catalogue of Agents and Their Target Weeds.* 4 CABI Publishing, Wallingford, United Kingdom.

Kissinger, D.G., 1966. *Cyrtobagous* Hustache, a genus of weevils new to the United States fauna (Coleoptera: Curculionidae: Bagoini). *The Coleopterist's Bulletin* 20: 125–127.

Landry, G.P., 1981. *Salvinia minima* new to Louisiana. *Amer. Fern J.* 68: 95.

Madeira, P.T., C.C. Jacono, P. Tipping, T.K. Van and T.D. Center, 2003. A genetic survey of *Salvinia minima* in the southern United States. *Aquat. Bot.* 76: 127–139.

Mitchell, D.S. and P.A. Thomas, 1972. The Kariba Weed: *Salvinia molesta*. *Br. Fern Gaz.* 10: 251–252.

Mitchell, D.S. and P.A. Thomas, 1972. The Taxonomy and Distribution of Salvinia Species in the Neotropics. *Ecology of Water Weeds in the Neotropics. Technicial papers in Hydrology 12.* UNESCO, Paris, France.

Sands, D.P.A. and M. Schotz, 1985. Control or no control: A comparison of the feeding strategies of two *Salvinia* weevils. In: *Proceedings of the VI International Symposium on Biological Control of Weeds*, Vancouver, Canada, August, 1984, Agriculture Canada, Toronto, Ontario, Canada. pp. 551–556.

Sands, D.P.A., M. Schotz and A.S. Bourne, 1986. A comparative study on the intrinsic rates of increase of *Cyrtobagous singularis* and *C. salviniae* on the water weed *Salvinia molesta*. *Entomol. Exp. Appl.* 42: 231–237.

Small, J.K., 1931. *Ferns of Florida: Being Descriptions of and Notes on the Fern-Plants Growing Naturally in Florida (illustrated)*. The Science Press, New York 190–193.

Swofford, D.L., 2001. *PAUP. Phylogenetic analysis using parsimony (and other methods). Version 4.0 Beta 10*. Sinauer, Sunderland, Mass.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 24: 4876–4882.

Tipping, P.W. and T.D. Center, 2003. *Cyrtobagous salviniae* (Coleoptera: Curculionidae) successfully overwinters in Texas and Louisiana. *Florida Entomol.* 86: 92–93.

Via, S., 1990. Ecological genetics in herbivorous insects: the experimental study of evolution in natural and agricultural systems. *Annu. Rev. Entomol.* 35: 421–446.

Walsh, B.D., 1864. On phytophagic varieties and phytophagic species. *Proc. Entomol. Soc. Phil.* 3: 403–430.